PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appl. No. : 10/812,170 Confirmation No. ~~1803~~ 3334

Applicant : Tadahiro HIRAMOTO et al.

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**DECLARATION UNDER 37 C.F.R. § 1.132**

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Tadahiro Hiramoto, declare and state:

1. THAT I attended Okayama University and received a Ph.D. degree in the study of the molecular mechanism of the interaction between barley and barley powder mildew in March 1993.

2. THAT since April 1993, I have been employed by Takasago International Corporation as a biochemist.

3. THAT I am one of the inventors of the antibacterial agent described and claimed in U.S. Patent Application Serial No. 10/812,170 and am aware that the claims of the application have been rejected as being obvious over JP H7-025764, identified by the USPTO

in the Office Action mailed August 25, 3005, as "Tamaoki et al.".

4. THAT to show that an antibacterial agent which is a mixture of nonvolatile compounds obtained by fractionation of the high-boiling point portion of a citrus cold press oil, the mixture containing 40% by weight or more of coumarin analogues, has unexpectedly superior antibacterial activity as compared to an antibacterial agent which is a mixture of nonvolatile compounds obtained by fractionation of the high-boiling point portion of a citrus cold press oil, the mixture containing less than 40% by weight of coumarin analogues, the following experiments were carried out under my direction and supervision.

[1] Example 1

The coumarin high concentration fractions (S-1 to S-4) and the low concentration fractions (C-3 and C-4) used in the tests for antibacterial ability in the examples and comparative examples in the present application were prepared by the following method:

1 Kg of citrus cold press oil derived from a lemon or a lime pericarp was placed in a heating container disposed in a distiller and gradually heated under reduced pressure. Volatile compounds were vaporized, liquefied in a cooler and accumulated in a receiving section. When the temperature of the citrus cold press oil in the heating container reached 120 C under pressure, the heating was

stopped. The amount of the residue (high-boiling point fraction) left in the heating container was 67 g.

A very small amount of ethyl acetate was added to 200 g of this high-boiling point fraction, which was then poured into a silica gel chromatographic column filled with 4 Kg of silica gel and the high-boiling pint fraction was carried on the silica gel.

Then, the fraction was eluted with 30 L of n-hexane to obtain a fraction 1. In succession, the fraction left in the column was eluted with a mixed solvent of ethyl acetate/hexane (volumetric ratio: 10:90), a mixed solvent of ethyl acetate/hexane (volumetric ratio: 20:80), a mixed solvent of ethyl acetate/hexane (volumetric ratio: 30:70), a mixed solvent of ethyl acetate/hexane (volumetric ratio: 50:50), and ethyl acetate 30 L each in volume to obtain a fraction 2, a fraction 3, a fraction 4, a fraction 5 and a fraction 6 respectively.

Each fraction was placed in an evaporator to emit the solvents thereby obtaining dry solids. The coumarin analogue content of each fraction was measured using the following method. 4 mg of the dry solid is dissolved in 50 mL of n-hexane or ethyl acetate and the solution was irradiated with ultraviolet light (wavelength: 311 nm) to measure the value of light absorption. The coumarin analogue content is shown as a relative value by applying the value of light

absorption to the density value of light absorption standard curve of coumarin (1,2-benzopyrone)-ethyl acetate solution and the percentage of coumarin analogue content in the dry solid is calculated. The percentage of coumarin analogue content in each fraction of lemon cold press oil was 0, 18, 87, 99, 99 and 63 % in order from fraction 1 to fraction 6. The percentage in each fraction of lime cold press oil was 1, 31, 100, 100, 91, and 89 % in order.

The dry solids from fractions 3, 4, 5, and 6, which contain a high concentration of coumarin analogues, were mixed. The resultant mixture is and called a coumarin analogue high concentration fraction. The coumarin analogue high concentration fractions derived from lemon cold press oil and lime cold press oil are S-1 and S-3, respectively. The coumarin analogue contents of S-1 and S-3 were 87.3 % and 96.1 %, respectively. Also, mixtures of the dry solids from the fractions 3 and 4 are called a concentrated fraction of the coumarin analogue high concentration fraction. The concentrated fractions derived from lemon cold press oil and lime cold press oil are S-2 and S-4, respectively. The coumarin analogue contents of S-2 and S-4 were 89.8 % and 100 %, respectively. Mixtures of the dry solids obtained from the fraction 1 and 2 contain a low concentration of coumarin analogues and are called a coumarin analogue low concentration fraction. C-3 and C-4 are the coumarin analogue low concentration

fraction derived from lemon cold press oil and lime cold press oil, respectively. The coumarin analogue contents of C-3 and C-4 were 9.6 % and 15.7 %, respectively.

C-1 is Trichlosane, which was purchased from TOKYO KASEI KOGYO CO., LTD. C-2 is butylparabene, p-t-butylbenzoic acid, which was purchased from Nacalai Tesque, Inc.

[2] Example 2

(1) Preparation of samples

The samples of each concentration (FR.1 to FR.5) were prepared by mixing S-3 obtained in Example 1 (coumarin analogue high concentration fraction derived from lime cold press oil) and C-4 obtained in Example 1 (coumarin analogue low concentration fraction derived from lime cold press oil). The coumarin analogue content of each samples were measured using the same manner as in Example 1. The results are shown in Table 1.

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Table 1.

| Sample No. | Mixed ratio | | Coumarin analogue content (% by weight) |
|---|---|---|---|
| | S-3 | C-4 | |
| FR.1 | 55 | 45 | 59.9 |
| FR.2 | 40 | 60 | 47.8 |
| FR.3 | 35 | 65 | 43.8 |
| FR.4 | 25 | 75 | 35.8 |
| FR.5 | 20 | 80 | 23.1 |

(2) Experimental Test: Measurement of Minimum Inhibitory Concentration (MIC)

These samples shown in Table 1 were dissolved in ethanol to prepare a serial twofold dilution stage and 100 µL of each was added to 10 mL of a sterilized agar medium [Mueller Hinton medium (Difco)], which was then stirred sufficiently, then transferred to a 9-cm-diameter Petri dish and solidified at ambient temperature. 5 µL of a diluted test bacteria solution was implanted in the Petri dish and cultured at 37 C for 72 hours. After the culturing was finished, the growth state of the medium in this Petri dish was compared with that in a Petri dish (blank) containing no sample and the concentration of the sample in which the growth of bacteria was not seen was defined as MIC. The results are shown in Table 2.

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As is clear from Table 2, The strength of antibacterial activity against the test bacteria was in order of FR.1 > FR.2 > FR.3 > FR.4 > FR.5. FR.1 to FR.3 had more effective antibacterial activity, however, FR.4 and FR.5 had not so or scarcely effective. These results shows that there is a close correlation between the strength of the antibacterial activity and coumarin analogues content and that the effective coumarin analogues content in fraction for antibacterial activity is more than 40% by weight.

Table 2.

| Bacteria | FR.1 | FR.2 | FR.3 | FR.4 | FR.5 |
|---|---|---|---|---|---|
| *Actinomyces naeslundii* | 25 | 50 | 100 | 1000 | >1000 |
| *Actinomyces viscosus* | 25 | 50 | 100 | 1000 | >1000 |
| *Fusobacterium nuclestum* | 12.5 | 25 | 50 | 500 | >1000 |
| *Porphyromonas gingivalis* | 12.5 | 25 | 50 | 500 | 1000 |
| *Prevotella intermedia* | 25 | 25 | 50 | 1000 | >1000 |

MIC:ppm

That all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and that further these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of

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Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent resulting therefrom.

Signed this 22 day of November, 2005.

Signed: Tadahiro Hiramoto

Name: Tadahiro HIRAMOTO